Improving the Understanding of Macro-Mechanics of Agarose and Collagen Hydrogels for Further Development in Regenerative Medicine

Undergraduate Thesis

Presented in partial fulfillment of the requirements for the Degree Bachelors of Science with Research Distinction in the Undergraduate School of The Ohio State University

By

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Abstract

Understanding material mechanical properties help materials scientists and engineers determine the best suited uses for a specific material. Engineering functional tissues in regenerative medicine is highly dependent on the structural and mechanical integrity of Extracellular Matrix (ECM). Currently scientists and engineers are looking to further their understanding of natural polymer responses to in vivo mechanical forces, such that these natural polymers can be utilized to engineer in vitro ECM models. This research focuses on (1) an analysis of mechanical properties of agarose and collagen hydrogels through uniaxial tensile testing and Rheometry testing and (2) effects of NIH3T3 fibroblasts on collagen hydrogels via gel contraction testing. Together, these mechanical factors give insight into how collagen would react to various mechanical stimulations found in an in vivo environment. Poisson's ratio, Young's modulus and shear stress were determined from stress and strain data collected during uniaxial and rheological testing. Gels at this time contained no cells. During time of mechanical testing a unique Poisson's Ratio verification technique was developed in order to eliminate the current deficiency of methodological verification in the materials science industry. Once material properties were determined and verified, cells were added to the collagen hydrogels to assess the effect of cells on mechanical properties of the hydrogels. Percent shrinkage was determined from gel contraction test image analysis. Normalized diameter data demonstrated expected shrinkage occurred over the course of 7 days. This experimental data can help to further develop the use of collagen hydrogels as a suitable material for engineering functional tissue in the regenerative medicine industry.

Acknowledgments

I would like to take this opportunity to express my gratitude to Dr. Heather Powell, Dr. Peter Anderson and David Gutschick for allowing me to be a part of this research project. I especially thank them for all of the help and support they have given me for the past 2 years as I was learning. Furthermore, I would like to thank all of the other faculty and graduate student researchers who have helped me through this venture through their teaching and support.

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Fields of Study

Major Field: Materials Science and Engineering

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Chapter 1: Introduction

A key component to engineering functional tissues is the structural and mechanical integrity of the extracellular matrix (ECM). The ECM plays a large role in directing new tissue synthesis which is why it is important that we strive to bridge the critical gap in our understanding of how the architecture and mechanics of the existing ECM controls how cells remodel and synthesize new matrix proteins. The primary objective of this study is to (1) develop a material platform where the ECM can be structurally and mechanically modified in a controlled manner and (2) further understand how cells modulate both native and naturally modified ECM. We used neutralized monomeric bovine dermal Type I collagen (Procol, Inamed Inc) and the collagen binding protein discoidin domain receptor 1 (DDR1) will serve as the ECM [1]. The DDR1 is used to disrupt the natural assembly process of Type I collagen gels. Preliminary research on the use of DDR1 as a collagen matrix modifier was conducted to show that DDR1 proteins do in fact affect the ultra-structure of collagen. Altered ultra-structure data will be used to support the macro-scale and micro-scale mechanical and architectural properties analysis of the modified ECM with and without cell population. The project focus is on a comparison between local environmental mechanical and structural properties on the micro-scale and average overall environmental, mechanical and structural properties on the macro-scale by using

the collected data to simulate local inhomogeneities and overall anisotropy of collagen hydrogels through a Finite Element Analysis (FEA). The two primary goals in this experiment are to study the effect of DDR1 and cell modulation to the collagen matrix. In order to understand these effects to the collage matrix, structural and mechanical property analysis will be performed. The hypothesis which we have determined for the aforementioned objectives are as follows:

(1) Relative strength and stiffness of the 3D collagen matrices and an increase in local mechanical inhomogeneity will result from the addition of DDR1. *However, as an external strain is applied to the modified 3D collagen matrix, mechanical inhomogeneity as well as anisotropy will increase [2].

(2) DDR1 modified collagen matrices will not alter cell traction forces. The macro-environment gel response will be dictated by the macro-mechanical properties of the matrix, while the micro-environment will be cell modulated in order to reduce inhomogeneities and balance the micro-mechanical environment will the cell traction forces [2].

Agarose Hydrogel experimentation (an isotropic material) was performed prior to collagen testing to solidify experimental procedures and to check consistency and validity of the macro-scale and micro-scale mechanical testing approaches. The macro-scale testing was done by performing traditional time-dependent uniaxial tension testing and rheological testing. Cell modulation effects on collagen hydrogels was studied via gel contraction tests. Incubating the neutralized monomeric bovine dermal Type I collagen (Procol, Inamed Inc) at a final concentration of 2.0 mg/ml at 37°C will in the desired

mold pattern will be done to form the collagen gels. DDR1 ECD (or the control protein TrkB; both from R&D Biosciences, MN) will be added to the collagen in 1:10 and 1:5 w/w ratio of DDR1 ECD to collagen [2]. Two collagen sample geometries will be used for the different testing apparatuses. The uniaxial testing sample will be executed with 4mm wide by 20 mm long dogbone samples and the rheological testing will be executed with circular samples formed in circular Teflon molds. A Bioreactor tensile testing will be used for the tension testing of the collagen gels. In addition to the macro-mechanical data, a novel two-point micro-rheometry approach, through the use of optic tweezers, will be done for micro-mechanical data collection (Physics). “The macro-mechanical uniaxial testing will furnish estimates of the mechanical environment in an average sense, across the entire tensile sample gauge section… The micro-mechanical testing will furnish estimates of the mechanical environment in a statistical sense and allow us to explore how the anisotropic and inhomogeneous nature of gels varies with macroscopic uniaxial strain and DDR1 concentration” [2]. Using this data, an FE model will be produced for analysis of the effect of DDR1 and strain on the 3D matrix. The modeling portion of this experiment will enable us to view the material characteristics of Type I collagen, including the anisotropic nature of ECM.

Chapter 2: Methodology

1 Phase I and II: Agarose and Collagen Mechanical Testing

The macro-scale mechanical testing was performed for varied concentrations of Agarose hydrogels as well as for Type I Collagen (Procol, Inamed,Inc) hydrogels with a final concentration of 1 mg/mL. All uniaxial mechanical testing is discussed in the following methodology, and Rheometry testing was performed by David Gutschick, PhD candidate.

1.1 Sample Preparation

All samples were prepared in a Teflon dogbone mold with dimensions 4 mm x 20 mm. Figure 1 displays the mold with set samples.

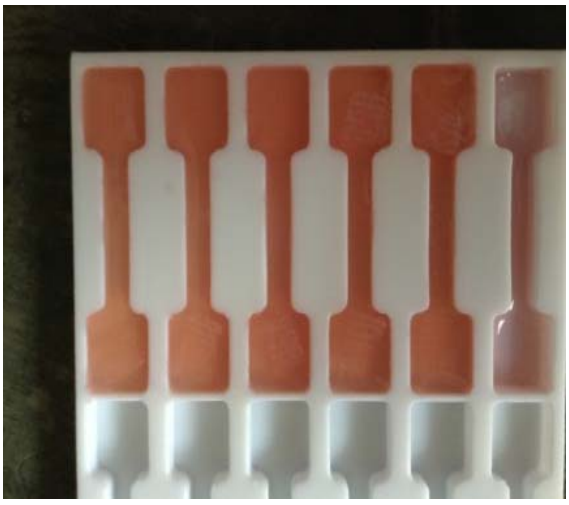


Figure 1: Teflon dogbone sample mold

1.1.1 Agarose

Agarose samples were prepared at varied concentrations and varied by Agarose type. The concentrations used during experimentation are listed in Table 1.

Table 1: Agarose varied concentration measurements

Precalc for 6 molds		
%	MilliQ (ml)	Agar
7	8.4	600
5	8.4	428.5714286
3	8.4	257.1428571
2.5	8.4	214.2857143
2	8.4	171.4285714
1.5	8.4	128.5714286
1	8.4	85.71428571
0.4	8.4	34.28571429

Agarose type varied by gelation temperature: low temperature (LT) or high temperature (HT). The HT agarose is indicated as such because a high temperature was required to maintain a low viscosity solution when creating the molds (i.e. the HT agarose would begin the gelation process at room temperature). The opposite is as such for the LT agarose, in that the LT agarose maintained its low viscosity at room temperature while formation of the samples was occurring. Both types of samples were prepared in the same fashion as listed in the following steps:

1. Measure agarose powder and MilliQ water necessary for desired concentration.
2. Place components in glass beaker on a hot plate with a magnetic stir bar.

3. Heat Agarose solution to ~90°C and allow the mixture to stir (~120 rpm) for approximately 30 minutes.
4. During solution mixing, place sterilized gauze pads in the sterile dogbone mold for better gripping when removing the samples at a later time.
5. Remove the Agarose solution from the hotplate and quickly fill each dogbone mold with 1.3 mL of solution.
6. Incubate the Agarose samples for 24 hours (For HT agarose, incubation is in fridge. For LT Agarose, incubation is in 37°C).

After the 24 hour incubation period, the Agarose samples were ready for testing.

1.1.2 Collagen

Collagen samples were prepared via the use of DME cell culture media, neutralized monomeric bovine dermal Type I Collagen (Procol, Inamed Inc) and sterile Sodium Hydroxide (NaOH). Table 2shows the measurements calculated for a desired collagen concentration of 1 mg/mL.

Table 2: Collagen hydrogel solution measurements

Media (mL)	Collagen (mL)	NaOH (mL)	Total (mL)	Desired []	Thickness (cm)	# of Dogbones
10.569	4.875	0.156	15.6	1	0.4	12

Collagen hydrogel samples varied only in some gels were made with suspended microbeads and some gels were made without microbeads. Microbead suspension was done to mimic sample types being made for micro-rheometry via optical laser tweezers

being done by as the micro-scale experimental procedures. The subsequent steps were followed in the fabrication of these hydrogels:

1. Place sterile gauze pads into dogbone mold ends for better gripping when removing the samples at a later time.
2. Keep Collagen, NaOH and 50 mL conical tube in an ice bath.
3. Dulbecco's Modified Eagle Medium (lifeTechnologies) (DME) cell media was placed in the 50 mL conical tube. (If using beads: microbeads were removed from solution via centrifuge and then re-suspended in the DME cell media)
4. NaOH was added to the media solution.
5. Collagen was very quickly added and mixed into the mixture.
6. Each dogbone mold was filled with 1.3 mL of solution.
7. Teflon mold was placed on a riser in a small culture dish and the bottom of the dish was filled with MilliQ water.
8. The dish was then covered, sealed and set in an incubator at 37°C to allow for gelation within a 24 hour time period.

It is important to note that the collagen gelation would begin to occur quickly; therefore, it was necessary to fill the molds quickly and not allow for excess time in the 50 mL conical tube.

1.2 Uniaxial Testing

All dogbone samples made underwent dynamic uniaxial mechanical testing. A bioreactor, pictured in Figure 2, utilizing Growth Works operating program was used to perform the uniaxial tensile tests.

Figure 2: Bioreactor for uniaxial tensile testing.

The bioreactor worked via a metal shaft connected to a solenoid motor which controlled the desired test motion. Growth Works operating system recorded the displacement of the sample as set by the user and the corresponding force associated with sample motion. The dogbone samples were removed from the mold and placed in the bioreactor for testing one at a time. Cyclical tests were performed for stress-strain analysis of each gel. Primary testing focused on utilizing displacement amplitude of 2 mm with a 2 Hz frequency. These parameters were chosen in order to match the micro-scale testing parameters.

2 Phase III: Collagen Gel Contraction Tests

Neutralized monomeric bovine dermal Type I Collagen (Procol, Inamed Inc) was used to create the collagen hydrogel samples for the gel contraction tests. A collagen

concentration of 2 mg/mL was used for all gels. NIH 3T3 fibroblasts were used as the cell line because fibroblasts are the main cell type that modulates collagen structure and mechanics within ECM. The NIH 3T3 fibroblasts were cultured and fed using supplemented DME (DM3). DME was supplemented with Fetal Bovine Serum (Life Technologies) (FBS) and Anitbiotic-Antimycotic (Life Technologies) (PSF).

2.1 Sample Preparation

NIH 3T3 fibroblasts were cultured in DM3 cell media until the cells reached 75% confluence. The cells were then harvested via trypsinization of the culture to remove the cells from the culture dish. Using a hemocytometer, the number of cells per 1 mL was counted and an appropriate volume of media for cell re-suspension was calculated. The desired density of cells used for these gel contraction tests was 500,000 cells per 0.500 mL of total collagen hydrogel solution in a singular well of a 24-well plate. Collagen gels were made in the same fashion as listed above; except the measurements were for a 2 mg/mL concentrated collagen hydrogel, as seen in Table 3, and DM3 cell media was used instead of the DME.

Table 3: Gel contraction 2 mg/mL collagen hydrogel solution measurements per well

DM3 (mL)	NaOH (mL)	Collagen (mL)
0.173	0.050	0.277

2.2 Gel Contraction Tests

Gel contraction tests were done to see the effects of cell modulation on the structural and mechanical properties of collagen hydrogels. Once samples were made, the collagen hydrogels would incubate at 37°C for 24 hours. The gels were then moved to a 6-well plate and photographed with a scale bar. This was day 00. Subsequent pictures were taken on the following days 03 and 06. Image analysis software (ImageJ, NIH, USA) was used to measure the well and gel diameter lengths. This data is presented in Chapter 3: Results and Discussion.

3 Other experimentation being performed by various project group members:

In addition to the macro-mechanical data, a novel two-point micro-rheometry approach, through the use of optic. Using this data, an FE model will be produced for analysis of the effect of DDR1 and strain on the 3D matrix. The modeling portion of this experiment will enable us to view the material characteristics of type 1 collagen, including the anisotropic nature of ECM.

Chapter 3: Results and Discussion

1 Mechanical Testing

Both agarose and collagen hydrogels underwent uniaxial mechanical testing. In Figure 1 both types of gels are depicted within the bioreactor set-up. It was observed that the difference in materials was quite striking. Collagen was much easier to handle when placing the gels into the test set-up because of the elastic properties of the collagen fibrils, while agarose proved to be a much more brittle material. Agarose lacks the fibrillin structure which is present in collagen.

Figure 1: Example of Agarose samples and collagen samples within the bioreactor.

The data collected from the Bioreactor testing was output data of gel displacement according to the motor location and the force associated with each displacement. From this information, stress-strain data was calculated and analyzed. It is important to note that only examples of agarose and collagen stress-strain data is presented in this report for various reasons discussed later in this section.

1.1 Agarose Mechanical Testing

Agarose is a non-fibrillin, isotropic structure which was used in this study to demonstrate the validity and consistency with experimental methodology, especially to act as a comparison between the macro-mechanical and micro-mechanical testing procedures. The effect of this type of composition is demonstrated in the difficulty in achieving accurate data from the bioreactor. The brittleness of the agarose hydrogels often caused pre-breakage points in the gels prior to any application of displacement. Macro-mechanical studies were done by analyzing stress-strain curves of agarose after cyclical uniaxial tensile testing. Figure 2 is an example of the stress-strain curve created as a result of displacement and force data collected from a 0.4% Agarose concentration dogbone sample.

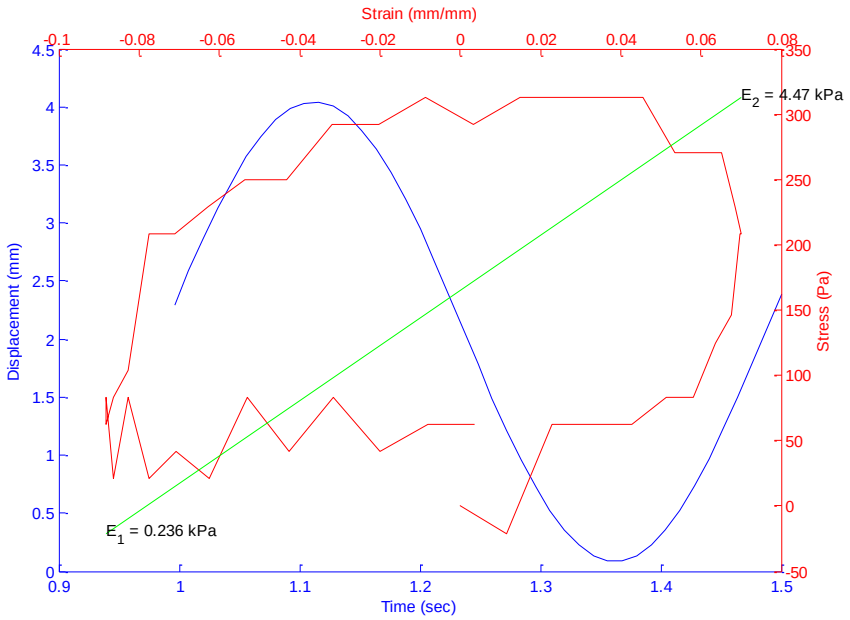


Figure 2: Example output of Agarose stress-strain curve

Each sample was evaluated at a singular period of the entire cyclical cycle so as to isolate the regions of data that were not conflicted by data from after the hydrogel had broken. The red axis is the stress versus strain data, the blue axis is displacement versus time and the green line is the average Young's Modulus calculated from the stress-strain data via Hooke's Law. The hysteresis demonstrates the irregularities in the registering forces as the bioreactor is collecting data. The jagged and sharp curvature means that the gel is either breaking during the run or slipping out of the bioreactor metal grips. Another contributing factor contributing to this error in data is mechanical issues with the bioreactor itself. These are discussed later on in this section.

Once motor issues were resolved, there were several data collections that demonstrated expected Young's Modulus values. These also matched the information being collected via micro Rheometry techniques. The LT agarose at 0.4% wt/wt concentration corresponded to the micro-Rheometry data which was ~2kPa. This data is presented in Table 1.

Table 1: Average data from 0.4% Agarose Trials

9-17-2013	9-23-2013	9-25-2013
E = 1.137 kPa	E = -0.099 kPa	E = 0.316 kPa
E' = 0.949 kPa	E' = -0.101 kPa	E' = 0.122 kPa
E'' = -1.813 kPa	E'' = -1.541 kPa	E'' = 0.572 kPa
E (slope) = 2.142 kPa	E (slope) = 1.919 kPa	E (slope) = 1.029 kPa

The LT agarose and the 0.4% concentration were deemed the most suitable material and concentration for further experimentation within this entire study due to these results. The matching data between the micro and macro mechanical testing was expected because of agarose's isotropy, thus helping to verify the methodology being utilized in the full study.

1.1.1 Poisson's Ratio Verification Method

The macro-mechanical testing data of agarose was further utilized to demonstrate the experimental verification of Poisson's ratio, Young's Modulus and Shear Modulus through the use of a video analyzer code and traditional uniaxial and Rheometry data collection. Current methods to define Poisson's ratio of a solid are often met with uncertainty. The Young's modulus (E), shear modulus (G), and Poisson's ratio (ν) of a solid are related by Equation 1, meaning that if two of the quantities are known, so is the third.

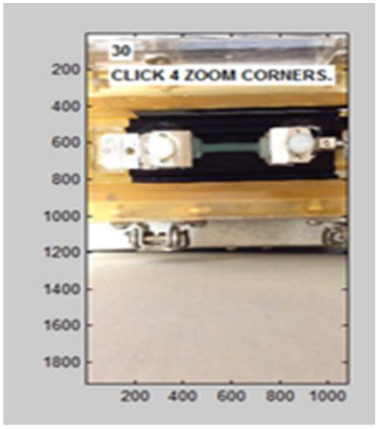
Equation 1: Relationship between Shear Modulus, Young's Modulus and Poisson's Ratio

$$G' = \frac{E'}{(2 + 2\nu)}$$

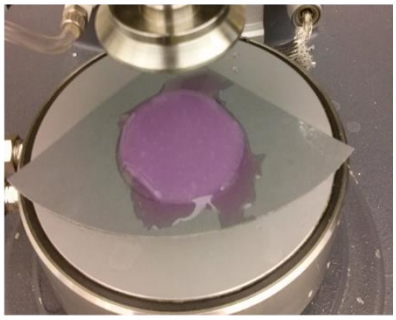
Measurement inaccuracies of E and G can inhibit the accuracy of a Poisson's ratio calculations. Currently, much of the literature involving these quantities presents results

without self-verification and makes the assumption that Poisson's Ratio is 0.5. The video analysis program was developed to determine Poisson's ratio directly by measuring transverse and axial strain of agarose and collagen hydrogels during uniaxial tensile testing. Measurement of all three properties provides a check of each measurement, validating both methodology and results.

Traditional Rheometry and uniaxial tensile test results were performed in order to find E and G. Force and displacement data used for E calculations. Figure 3 shows the experimental set-up of each testing procedure.



Sample gel in Bioreactor Chamber prepared for testing via a Trapezoid tensile test.



Rheological data collection set-up.

Figure 3: Bioreactor set-up for uniaxial tensile testing and Rheometry set-up for shear stress data collection.

The same uniaxial tensile tests used for the collection of Force and displacement data for calculation of E were recorded via IPhone video camera and then used in the video analyzer code developed by David Gutschick via MATLAB. Figure 4 depicts the operations of the video analyzer code.

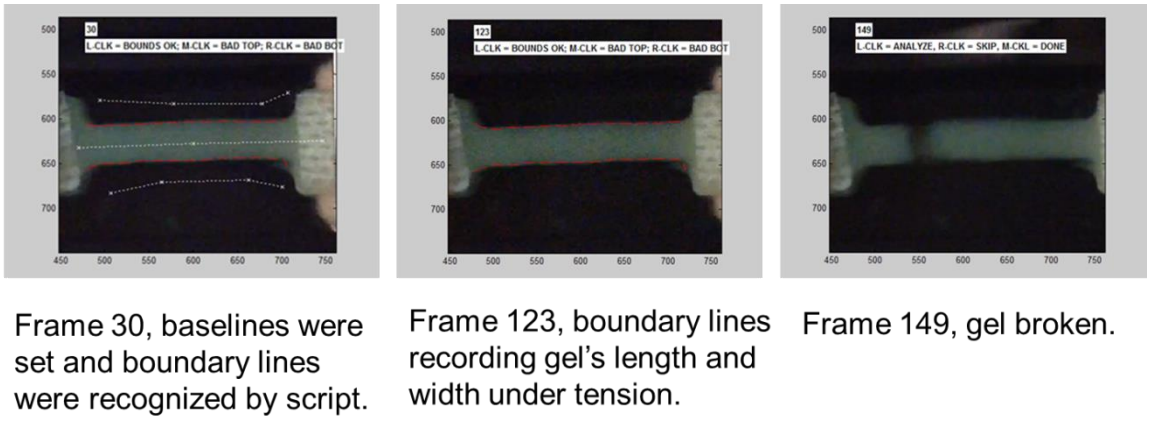


Figure 4: Video analyzer frame-by-frame measuring of gel length.

The code functioned as follows:

1. A beginning frame was chosen close to the start of the run. Within this frame, baselines and boundary lines were set around the gel for the code to recognize what lengths were being measured.
2. We would then click through the video frame-by-frame, thus changing the length of the gel. The boundary lines would recognize the change in length of the gels and collect this data.
3. This process would continue until the first frame of the broken gel appeared.

From these analytics and data collection, the transverse and axial strain could be measured and Poisson's Ratio calculated via the inverse relationship between transverse and axial strain. Figure 5 depicts the transverse strain versus axial strain graph, with Poisson's Ratio as the slope of the curve, of a 1% Agarose gel.

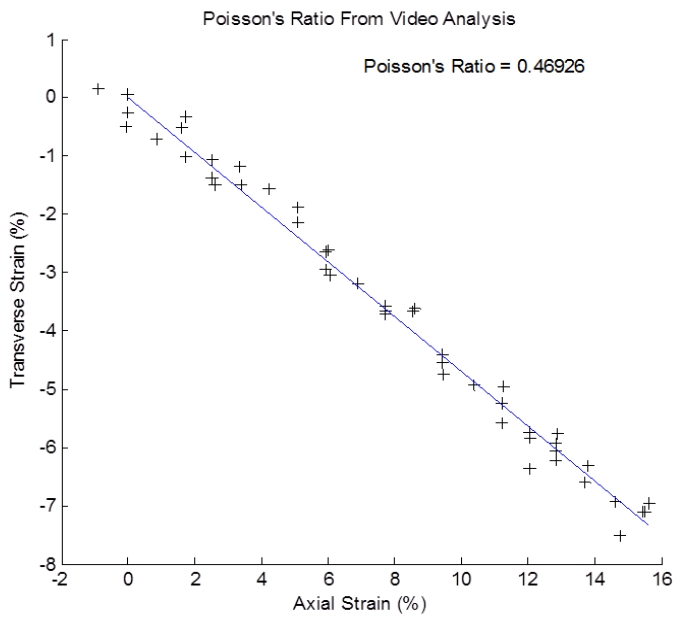


Figure 5: Transverse Strain versus Axial Strain calculations using video analyzers

Poisson's ratio from the video analyzer techniques was compared to the E and G calculated Poisson's ratio. Data for a 1% agarose gel is depicted in Table 2.

Table 2: 1% agarose properties at 2 Hz frequency

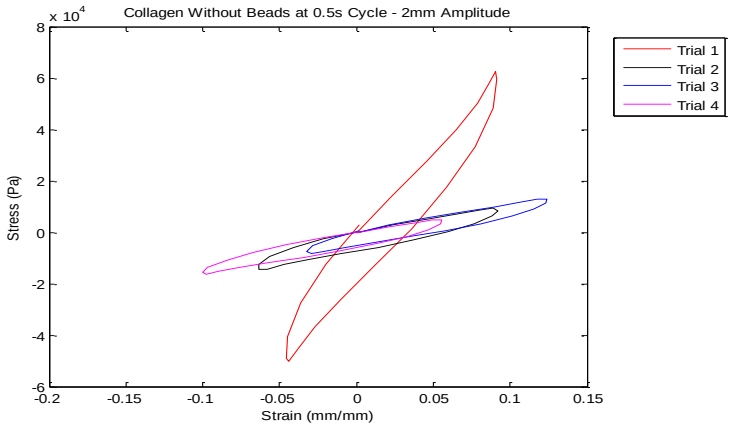
	G' (Pa)	G" (Pa)	E' (Pa)	v
1% Macro	3912±75	156±10	12220±4587	0.56±0.21 (G,E) 0.48±0.05 (img)

Our results for both agarose and collagen (from the multi-verification method) correspond with literature values. These verification techniques demonstrate the accuracy of our results and testing methods.

1.2 Collagen Mechanical Testing

Collagen is a widely studied material, utilized to learn more about ECM mechanics and structural composition as well as ECM cell modulation. Similar to the aforementioned agarose hydrogels, collagen hydrogels too underwent uniaxial testing. The data observed was again disrupted by bioreactor mechanical issues; however, Figure 6 depicts stress-strain curves from some collagen sample tensile tests.

A



B

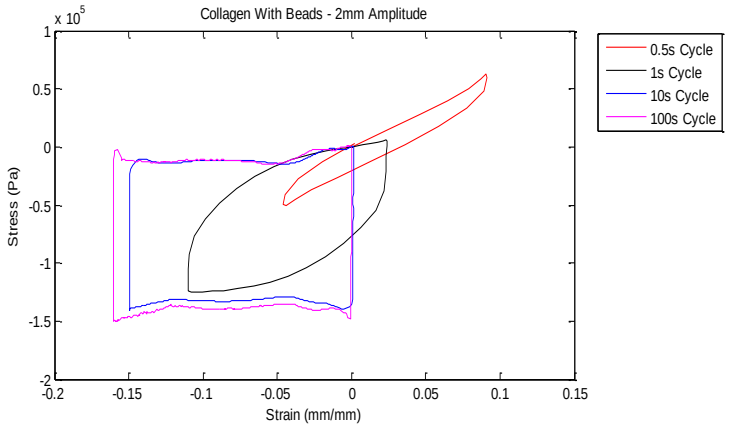


Figure 6: Summary Collagen stress-strain results. (A) Collagen samples made without microbeads run at a 2 Hz frequency and a 2 mm amplitude. (B) Collagen samples made with microbeads run at various frequencies and a 2 mm amplitude.

We found that the collagen data had a much more positive response to tensile testing, mostly likely due to the ease of handling of the collagen gels and the elasticity of the collagen hydrogels. The elasticity is a result of the aligning of the collagen fibrils as tension is applied to the gels. There was much less breakage during the testing, and therefore the resulting stress-strain curves are much smoother. Note that in Figure 6B, only the red curve sample was run at a 2 Hz frequency and will therefore be the only curved used in the bead comparison. Furthermore, the other stress-strain curves in Figure 6B are representative of trial runs at lower frequencies. We believe that the reasoning behind the square curves is due to undesired friction of created from poor shaft alignment in the bioreactor.

Figure 6A is depicting stress-strain curves of collagen hydrogels fabricated without the incorporation of microbeads and Figure 6B is depicting stress-strain curves of collagen hydrogels fabricated with the incorporation of microbeads. The collagen hydrogels with microbeads demonstrated stresses an order of magnitude higher than those fabricated without microbeads. This is due to the microbeads creating a stiffer matrix 3D collagen matrix.

1.3 Bioreactor Trouble Shooting

As stated previously, much of the tensile testing data was adversely affected by mechanical issues the bioreactor. There were both motor issues and hardware alignment issues that could have been causing excess friction and incorrect data recording. Figure 7

depicts (1) a graph of force versus time for two different bioreactor motors and (2) the shaft alignment system which connects the bioreactor grips, where the sample is placed, to the solenoid motor.

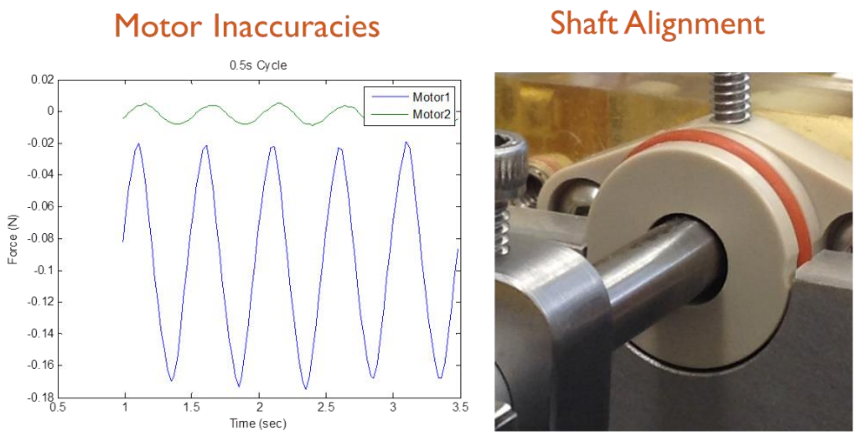


Figure 7: Problems with bioreactor

The motor inaccuracy graph was created via running both motors with the bioreactor without a gel set in the bioreactor; therefore, the expected results should be a very low amplitude sine wave oscillating around 0 N. However, Motor 1 (which was being used during data collection) shows a large sine wave oscillating around approximately -0.1N. This inaccurate reading led to many of the inaccurate force readings collected during the agarose and collagen uniaxial tensile tests. The poor force readings could also have been because of extra frictional forces caused by an unaligned shaft connecting the dynamic metal grip of the bioreactor and the motor.

2 Gel Contraction Test

Gel contraction data was collected to observe the cell modulation of collagen hydrogels. Previous studies have shown that a collagen hydrogel will contract naturally without cell modulation, but when NIH 3T3 cells were added in the collagen hydrogel, the gels demonstrated even more contraction. Figure 8 shows images of the gels taken with an IPhone camera.

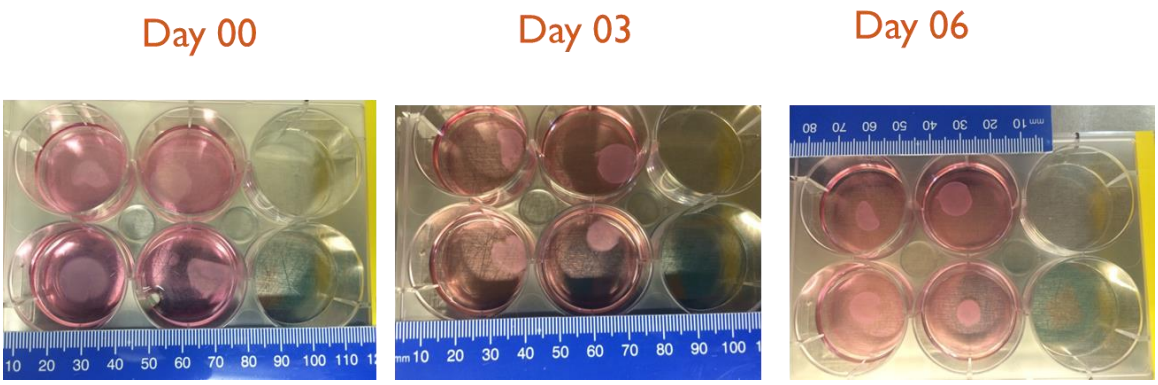


Figure 8: Days 00, 03 and 06 during collagen gel contraction tests

It can be seen that the size difference between day 00 and day 06 are fairly significant, which is what would be expected of the hydrogels with the addition of cells.

The size of the gels was quantified via normalization of the gel diameters as compared to the size of the well via Equation 2.

Equation 2: Normalized diameter calculations

$$\frac{Gel\ Length}{Well\ Length}$$

This analysis was performed using image analysis software (ImageJ, NIH, USA). Figure 9 is a schematic explaining how the measurements of the diameters were taken. This approach was used to eliminate any data discrepancies as a result of picture warping.

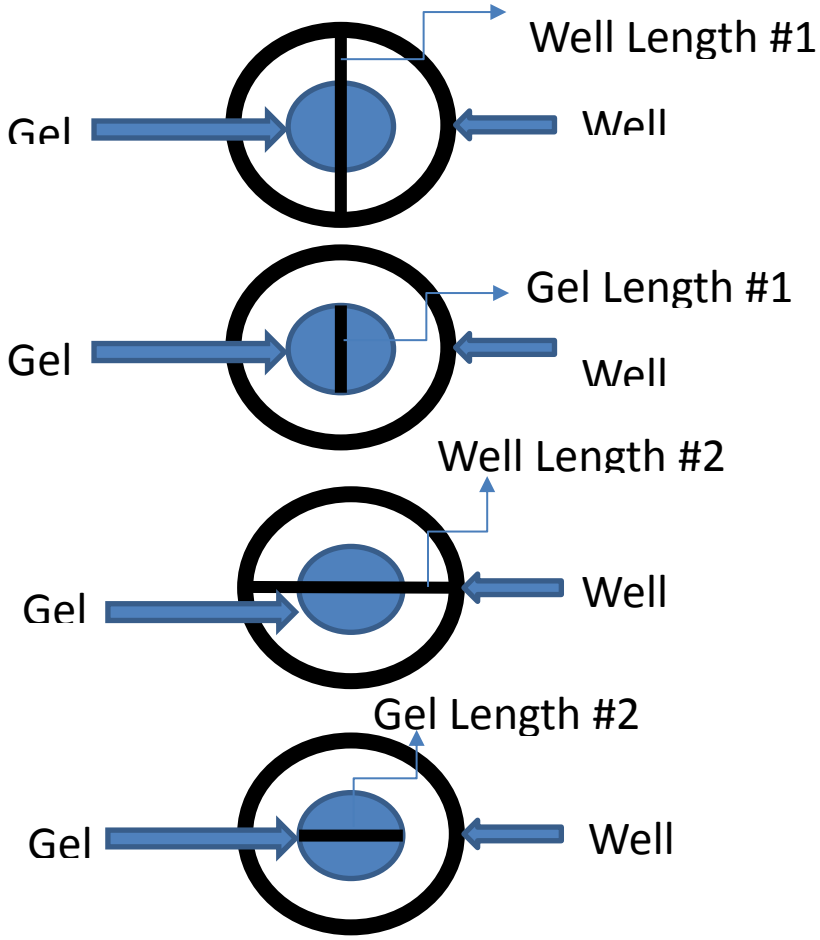


Figure 9: Gel contraction diameter measurements

As expected, the data collected is indicative of a hydrogel with a significant decrease in area. Figure 10 demonstrate the results of the normalized data.

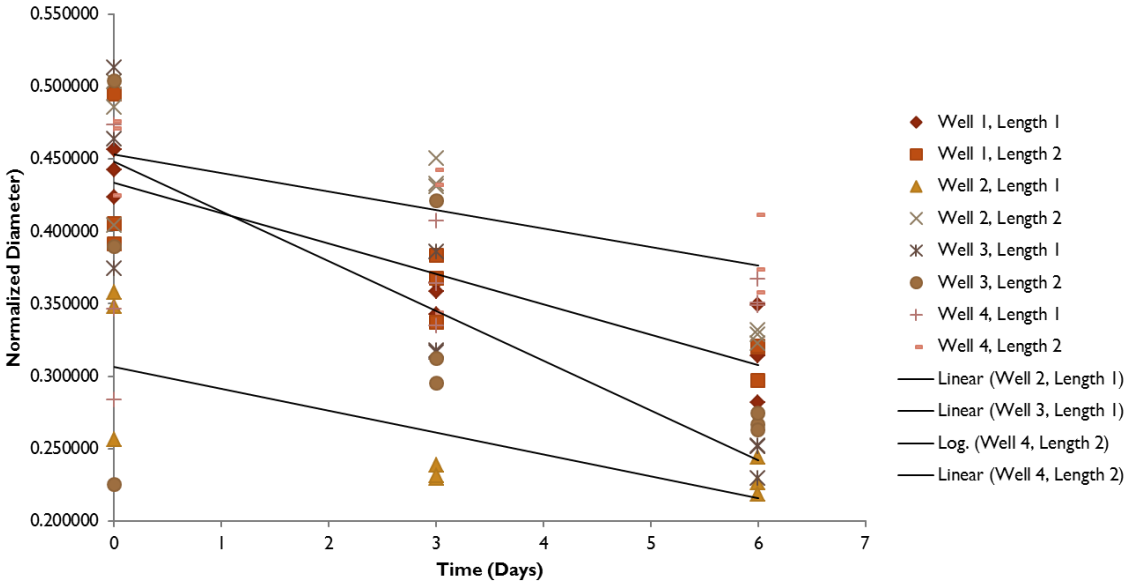


Figure 10: Normalized diameter according to days

This decrease over time is partially due to the modulation of the hydrogels by the fibroblast cells. This is due to the cell movements and attachments to the collagen fibers within the hydrogel. The cell mechanics results in a restructuring and constriction of the structural properties of collagen fibers.

Chapter 4: Conclusion

Agarose hydrogels were used to finalize and verify the multiple micro-scale and macro-scale mechanical testing procedures utilized throughout the greater portion of this project. The isotropy of agarose proved to be favorable for these purposes because the (1) many previous studies have been done on agarose and (2) agarose comparison results were predicable. These verifications of our methodological approach allowed us to utilize these methods for the testing of collagen hydrogels. Collagen is a material that can be widely used for biomaterial applications due to its natural biological composition, which contributes to its high biocompatibility properties. Further work to be included within this study includes the further macro-mechanical data collect to correct for all of the inaccuracies due to bioreactor mechanical issues. This data can then be used to make more comparisons to the (1) micro-mechanical experimental data and (2) the FEM analysis of micro-mechanical and structural responses to various applied forces. Furthermore, the addition of DDR1 should be incorporated into all levels of experimental testing. The addition of DDR1 will allow further research and understanding of natural modifiers of collagen and the mechanical and structural responses in relation to cell modulation.

The study of these factors is key concepts to understanding the principal material characteristics of collagen as a biomaterial. Through a better knowledge of the simple

structural and mechanical properties of the collagen, a primary structural component in natural tissue ECM, scientist, engineers and doctors will be able to optimize use of collagen for tissue engineering or drug delivery techniques. This study also eliminates the assumed presuppositions made about collagen. In other words, we no longer can make the assumption that collagen is a homogenous 3D matrix. But if researchers are able to understand the anisotropy of the collagen local and global mechanical and structural properties, then they will be able to use that technology to further enhance regenerative and personal medicine. This study is important to our fundamental understanding of natural polymers for their uses as biomaterials.